MECHANISM OF THE DISSIMILATION OF VOLATILE ORGANIC ACIDS BY METHANOGENIC ENRICHMENTS

By

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To Jane.

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Methanogenic enrichments were examined for evidence that molecular hydrogen is an intermediate in the production of methane from acetate, propionate, and butyrate. Large volumes of 100% CO_2 were bubbled through these enrichments to flush out dissolved gasses produced during dissimilation of these substrates. The gas removed from the enrichments was then concentrated by removal of CO_2 using alkali and analyzed for methane and hydrogen. Large amounts of hydrogen were produced by enrichments utilizing propionate or butyrate, but not enrichments utilizing acetate only. Propionate and butyrate enrichments were also shown to have the ability to utilize externally supplied molecular hydrogen in large amounts with no lag. Acetate enrichments utilized hydrogen to a lesser degree. Hydrogen oxidizing methanogenic bacteria were present in numbers exceeding 10^8 per ml in cultures enriched with propionate or butyrate, and about ten-fold fewer in an acetate enrichment. No hydrogen oxidizing isolate was able

to utilize propionate or butyrate under culture conditions used. Thus, large amounts of hydrogen are produced during dissimilation of propionate or butyrate, and large amounts of hydrogen can be oxidized in the production of methane by these enrichments. Since methane producing bacteria seem to be unable to dissimilate propionate and butyrate, a non-methanogenic bacterium may oxidize propionate or butyrate with the production of hydrogen. This hydrogen would subsequently serve as a methanogenic substrate for methane producing bacteria present in the enrichment.

INTRODUCTION

The biological formation of methane gas is all but ubiquitous in anaerobic environments where light and inorganic oxidants such as sulfate and nitrate are limiting (32). The ultimate products of dissimilation of organic matter under these conditions are methane and carbon dioxide. This conversion is thought to occur in two steps (19, 29). The first is the breakdown of complex organic matter to molecular hydrogen, carbon dioxide, and volatile organic acids (VOA), predominantly acetic, propionic, and butyric. These intermediates are converted to methane and carbon dioxide in the second step. Since an accumulation of these intermediates is often associated with digestor failure in anaerobic waste treatment processes (24), an understanding of the nature of the conversion of these intermediates to terminal products is therefore a necessary prerequisite to optimal waste treatment design.

Conversion of the gaseous intermediates (hydrogen and carbon dioxide) to methane is performed by all known species of methanogenic bacteria (5).

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad (1)$$

Recent evidence has indicated that methanogens, though they resemble other bacteria in many ways, are so unique as to warrent consideration as a class of life distinct from prokaryotes and eukaryotes (17). Methanogens which have been examined for the presence

of peptidoglycan in their cell walls have been found to lack it (14). Coenzymes (2-mercaptoethanesulfonic acid and $Factor_{420}$) present in all methanogenic bacteria examined have been found in no non-methanogenic organisms. These coenzymes are so unique that one, $Factor_{420}$, has been used as a distinguishing characteristic in the preliminary identification of methanogenic colonies (10). A third distinction is the mechanism of assimilatory carbon dioxide reduction. Autotrophic methanogens lack ribulose 1,5-biphosphate carboxylase, an enzyme essential to the Calvin cycle, previously considered necessary for autotrophic carbon dioxide assimilation (32). Finally, ribosomal RNA treated with T_1 ribonuclease gives products substantially different from other bacteria, indicating an evolutionary divergence of methanogens from the main bacterial line which significantly antedates the divergence of the *Cyanophyta* (1, 11).

Much of the biochemistry of the conversion of hydrogen and carbon dioxide to methane has been elucidated in the laboratory of Ralph Wolfe and others (4, 8, 18, 22, 29-32). An understanding of the conversion of volatile organic acids to terminal products has been more elusive. Van Niel's Carbon Dioxide Reduction Theory of methanogenesis proposes that all methane produced in anaerobic fermentations is the result of the reduction of carbon dioxide (2). According to this theory all intermediates must be oxidized to carbon dioxide, and the ultimate fate of electrons generated by this oxidation is the reduction of carbon dioxide to methane.

Radioactive tracer experiments have demonstrated that acetate dissimilation is probably an exception to the Carbon Dioxide Reduction Theory. Carbon-14 (7, 26) and deuterium (20) labeling experiments have indicated that the methyl group of acetate is converted directly to methane with its hydrogens intact:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2)$$

Tracer studies of methanogenic enrichments have shown that acetate is an intermediate in the methanogenic dissimilation of propionate and butyrate (13, 26). If one assumes that the Carbon Dioxide Reduction Theory applies to these conversions, they can be rewritten as the sum of an oxidation reaction and a reduction (methanogenic) reaction.

For propionate:

$$4CH_3CH_2COOH + 8H_2O \rightarrow 4CH_3COOH + 4CO_2 + 24H^+ + 24e^- \quad (3)$$

$$24H^+ + 24e^- + 3CO_2 \rightarrow 3CH_4 + 6H_2O \quad (4)$$

$$4CH_3CH_2COOH + 8H_2O \rightarrow 4CH_3COOH + CO_2 + 3CH_4 \quad (5)$$

And for butyrate:

$$2CH_3(CH_2)_2COOH + 4H_2O \rightarrow 4CH_3COOH + 8H^+ + 8e^- \quad (6)$$

$$8e^- + 8H^+ + CO_2 \rightarrow CH_4 + 2H_2O \quad (7)$$

$$2CH_3(CH_2)_2COOH + 4H_2O \rightarrow 4CH_3COOH + CH_4 \quad (8)$$

Stadtman and Barker demonstrated that in the presence of $H^{14}CO_3^-$, the

methane produced in reactions 5 and 8 has the same specific activity as bicarbonate, indicating that all methane was produced by carbon dioxide reduction (27). These experiments were performed with highly purified cultures which were unable to convert acetate to methane and carbon dioxide. The cultures have been lost and never re-isolated; they are now believed not to have been pure cultures. No existing pure cultures of methanogenic bacteria have been demonstrated to be capable of utilizing propionate or butyrate as a methanogenic substrate (5).

The mechanism of the methanogenic oxidation of propionate and butyrate was thought to occur in a manner analogous to the methanogenic ethanol oxidation of the now defunct *Methanobacillus omelianskii*. This culture oxidized ethanol to acetate while reducing carbon dioxide to methane (3, 25). Ferredoxin was thought to be the electron carrier involved, and in the absence of carbon dioxide, reduced ferredoxin was oxidized by a hydrogenase which produced molecular hydrogen. This culture was also able to use molecular hydrogen for the reduction of carbon dioxide.

M. omelianskii has since been found to be an association of two species of bacteria, *Methanobacterium* Strain M. o. H. and S organism (5). S organism is capable of oxidizing ethanol to acetate with the production of hydrogen (21). Hydrogen inhibits this oxidation, and thus when it is grown on ethanol in pure culture, hydrogen quickly accumulates and inhibits further acetate production.

Strain M. o. H. converts hydrogen and carbon dioxide to methane according to reaction 1, but is unable to catabolize ethanol. When the two organisms are co-inoculated into media containing ethanol and carbon dioxide, large quantities of methane and acetate are formed. The resolution of this association of bacteria brings up the important point that hydrogen can function as an intermediate even when present at concentrations too small to be detected.

Other examples of the transfer of molecular hydrogen to methanogenic bacteria are provided by organisms which alter end products of carbohydrate fermentations in the presence of methanogenic bacteria (16). *Ruminococcus flavefaciens* produces formate, acetate, succinate, hydrogen, and carbon dioxide when grown on cellulose (15). When co-inoculated with *Methanobacterium ruminantium* there is a shift in products to more oxidized states: Succinate production decreases and acetate production increases. No hydrogen is detectable in the final products, but more hydrogen is produced (based on methane production) than is produced by the pure culture of *R. flavefaciens*. Apparently the maintainance of low hydrogen concentrations by the methanogenic bacterium allows increased hydrogen production by the non-methanogen.

In pure culture *Clostridium cellobioparum* converts glucose to acetate, ethanol, formate, and hydrogen; when co-inoculated with *Methanobacterium ruminantium* its major end products are acetate and methane (9). A 22% increase in the yield of *C. cellobioparum* per mole of glucose indicates that in the presence of the hydrogen

oxidizing methanogen, more complete glucose oxidation allows a higher molar energy yield.

Selenomonas ruminantium ferments carbohydrates predominantly to lactate, propionate, acetate, and carbon dioxide (23). Several strains have been shown to produce small amounts of hydrogen. The production of hydrogen (calculated from the production of methane) increases markedly when its concentration is maintained at low levels by co-inoculating with a methanogenic bacterium.

These examples show that interspecies transfer of molecular hydrogen may be an important source of reducing power for the reduction of carbon dioxide in methanogenic fermentations. It is the purpose of this study to determine whether interspecies hydrogen transfer plays a role in the dissimilation of propionate and butyrate. If one rewrites the oxidation half-reactions for propionate and butyrate dissimilation (reactions 3 and 6) as molecular hydrogen producing reactions and calculates the free energy change, one sees a reason for the rejection of this as a possible mechanism.

$$4CH_3CH_2COOH + 8H_2O \rightarrow 4CH_3COOH + 4CO_2 + 12H_2 \qquad \Delta G^{\circ\prime} = +18.2 \text{ kcal/mole prop.} \qquad (9)$$

$$2CH_3(CH_2)_2COOH + 4H_2O \rightarrow 4CH_3COOH + 4H_2 \qquad \Delta G^{\circ\prime} = +11.5 \text{ kcal/mole but.} \qquad (10)$$

The positive standard free energy change (28) for these reactions makes these hydrogen producing reactions appear unlikely. However, conditions common to anaerobic digestion are radically different from standard conditions used for calculation of standard free energy changes.

A partial pressure of one atmosphere of hydrogen is used for calculation of $\Delta G^{\circ\prime}$, although hydrogen is ususlly undetectable in gas produced from active digestors. As hydrogen partial pressure decreases, ΔG decreases. At 37°C and partial pressure of carbon dioxide at 0.3 atm, the production of molecular hydrogen from propionate (reaction 9) is thermodynamically spontaneous when the partial pressure of hydrogen is less than 5.4×10^{-5} atm, and the production of molecular hydrogen from butyrate (reaction 10) is spontaneous at partial pressures of hydrogen less than 1.6×10^{-4} atm. If this is indeed the mechanism for propionate and butyrate dissimilation, then an important function of methanogenic bacteria in anaerobic digestion is the maintainance of low partial pressures of hydrogen which permit the rapid oxidation of toxic volatile organic acids by other bacteria.

MATERIALS AND METHODS

Construction of Anaerobic Filters

Three laboratory scale anaerobic filters were fabricated from 6 inch diameter polyvinyl choloride (PVC) pipe. The pipe was cut 6 to 7 feet in length and capped with a PVC cap at the bottom. Holes were drilled into the pipe at one foot intervals for sampling ports. A 5/8 inch PVC pipe threaded at the distal end was glued into each hole. Plastic tubing was inserted through one hole stoppers and placed in the end of each sampling port so the tubing protruded about 5 cm into the filter. Stoppers were secured with threaded PVC caps in which a hole had been drilled through which the plastic tubing protruded. Short lengths of latex tubing were pushed over the external ends of the plastic tubing and sealed with pinch clamps.

The filter was then filled with rock (hand sized at 3 to 4 cm diameter) and gassed out with a 70% nitrogen: 30% carbon dioxide gas mixture, and filled with inoculum. The top of the filter was then capped with a PVC cap, and a port was fabricated in the top through which effluent flowed. Feed solutions entered at the bottom port of the filter and flowed upwards. Liquid and produced gas were separated by an inverted siphon, gas production being measured by a Wet Test gas meter or by water displacement.

Preparation of Enrichment Cultures

Anaerobically digested domestic sewage sludge obtained from the Gainesville, Florida, Wastewater Treatment Plant was used as an inoculum for the first filter. Sludge was collected and handled in insulated carriers to protect organisms from temperature shocks. The filter was fed various complex organic wastes for two years, and in November of 1975 material was withdrawn from the bottom port of the filter to serve as inoculum for each of the two other filters. Since that time, the sole carbon and energy source supplied to each filter has been a single volatile organic acid (acetic, propionic, or butyric). A dilute salt solution (20 mg/l NaCl, 20 mg/l $NH_4H_2PO_4$, 4 mg/l $MgCl_2 \cdot 6H_2O$, 3 mg/l NH_4Cl, 1 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.1 mg/l $(NH_4)_2SO_4$) constituted the bulk of the feed solution. A concentrated feed solution (consisting of a mixture of calcium and sodium salts of the volatile organic acid) commingled with the dilute salt solution as it entered the filter. The ratio of calcium to sodium salt was determined empirically to give a pH of 6.8 to 7.0. Sodium salts would have raised the pH because the products of fermentation are gasses (methane and carbon dioxide), Na^+ and OH^-. When calcium salts are fermented, calcium ions precipitate as $CaCO_3$ with no net alkaline production. However, a certain amount of sodium salts must be included to neutralize dissolved carbon dioxide and volatile organic acid pools.

Anaerobic Digestor

An 8 l fermentor bottle served as a laboratory scale anaerobic sewage sludge digestor. Anaerobically digested domestic sewage sludge obtained from the Gainesville, Florida, Wastewater Treatment Plant served as an inoculum. The sludge was collected and handled in containers previously gassed out with oxygen-free gas. The laboratory scale digestor was maintained at 35°C in a water bath, and was mixed by stirring 15 min every hr. Each day 250 ml of sludge were removed while stirring, and 250 ml of raw sludge added. The raw sludge was obtained from the Gainesville plant, blended in a Waring blender for 5 min, and stored frozen in polypropylene bottles. Gas production, measured by displacement of water, increased slightly over the first week of incubation and leveled off at 4.2 to 4.8 l per day; the gas was composed of 63 to 72% methane and 30 to 35% carbon dioxide.

Gasses

All gas was obtained from the Matheson Gas Company. Gas mixtures for routine digestor, manometry, and culture work were Purified grade. Traces of oxygen were removed by passing gasses over freshly reduced hot copper filings. Carbon dioxide for sparging experiments (Anaerobe grade) was passed through two gas scrubbers containing 500 ml of 0.018 M titanous chloride solution which removed traces of oxygen and saturated the gas with water. Gasses for gas-liquid chromatography were Purified grade and those used as standards were Ultra-high purity.

Determination of Volatile Organic Acids and Radioactivity in Volatile Organic Acids

Volatile organic acids were quantitated using a Packard 800 Series gas chromatograph. A 6 foot by 2 mm glass column was packed with 10% SP1000 (Supelco, Bellefonte, Pa.) and 1% phosphoric acid coated on 70/80 mesh Anakrom AW (Supelco). Temperatures were inlet 150°C, column 122°C, detector 132°C, and outlet 148°C. Carrier gas (100% nitrogen) flow rate was 25 ml per min. Detection was by flame ionization; flow rates to the detector were 30 ml of hydrogen per min and 300 ml of air per min. The signal from the detector was connected to an Autolab Minigrator which was used to quantitate by comparison with standards. Samples were prepared for analysis by mixing 0.9 ml of sample with 0.1 ml of 30% w/v phosphoric acid, followed by centrifugation to remove cells. Injection volume was 0.4 µl.

When quantitation of radioactivity of volatile organic acids was desired, injection volume was increased to 4 µl and a stream splitter (ratio ca. 18:1) was inserted in the line of the column effluent, so that one part of the effluent went to the detector and eighteen parts were diverted to a port protruding through the column oven wall. Pasteur pipets were lightly packed with glass wool and a through-hole septum was placed in the top. Just prior to use the glass wool was wet with a solution of 1 N KOH in anhydrous ethanol. The septum was inserted onto the side port of the chromatograph just prior to the time the desired volatile organic acid was eluting from the column and removed when the elution was complete. The acid trapped on the alkaline

glass wool was washed into a scintillation vial with 10 ml of scintillation fluid (4 g 2,5-Diphenyloxazole, 0.1 g 1,4-bis-[2-(5-Phenyloxazolyl)]-benzene, and 250 ml of dry ethanol per liter of scintillation grade toluene). The amount of volatile organic acid diverted to the detector was quantitated to ditermine by subtraction the amount sent to the collector. Recovery of standard solutions of labeled volatile organic acids was greater than 90%. Samples were counted in a Beckman LS-133 Liquid Scintillation System. Counting efficiency was greater than 90%. Six replicate samples were analyzed and averaged for radioactive volatile organic acid determination.

Manometry

All manometric experiments were performed in 100 ml Warburg flasks having a single side arm fitted with a septum. An 18 ga needle was used to pierce the septum to allow air to exit as the flasks were gassed out with oxygen free gas. Septa were removed for introduction of enrichment cultures which were transferred in volumetric pipets while maintaining anaerobic conditions. The septa were immediately replaced, still transversed by the needles, while gassing continued. After 5 min the enrichment cultures were shaken at a rate of 70 strokes per min for 5 min to allow equilibration of the gas with the liquid. Needles were then removed, the flasks equilibrated to atmospheric pressure and sealed, ant the experiments were begun. To change atmospheres in the vessels during an experiment, needles were re-inserted through the septa and the flasks gassed out with the second gas mixture.

For volatile organic acid analysis, liquid samples of 0.9 ml were removed by syringes previously gassed out with oxygen free gas.

Collection of Sparge Gasses

Effluent gas from sparging experiments consisted of nearly 100% carbon dioxide. Large volumes of gas were absorbed quickly by bubbling through NaOH solution in a one liter Erlenmeyer flask. The flask was sealed with a three-hole stopper. The sparge gas entered through one hole via a glass tube reaching to the bottom of the flask. As the gas bubbled up, liquid was displaced through another glass tube reaching to the bottom of the flask and connected to a reservoir containing NaOH solution. The third hole was filled by a short piece of glass tubing sealed at the top by a septum through which alkaline insoluble gasses could be removed by a syringe at the end of an experiment. At very high sparge rates the flask was placed on a reciprocal shaker to increase the rate of carbon dioxide absorption. At the end of the collection period the flask was shaken 5 additional minutes to allow complete absorption of the carbon dioxide.

Culture Techniques

The modified Hungate technique (12) was used for isolation of strict anaerobes from enrichment cultures. All media used for isolation of and growth of pure cultures was 4 g/l $NaHCO_3$, 0.5 g/l KH_2PO_4, 1.0 g/l NaCl, 0.5 g/l NH_4Cl, 0.5 g/l $CaCl_2 \cdot 2H_2O$, 0.5 g/l L-cysteine-HCl, 50 mg/l $MgCl_2 \cdot 6H_2O$, 10 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l resazurin, and 30% fresh bovine rumen fluid. Just prior to use of media, 200 mg/l $Na_2S \cdot 9H_2O$ were added. The gas phase was either 70% nitrogen:

30% carbon dioxide or 70% hydrogen: 30% carbon dioxide. Solid media was made by adding 1.5% Ionagar #2 (Colab Laboratories, Glenwood, Ill.).

Quantitation of Radioactivity in Gasses

A 250 ml Cary-Tolbert Ionization Chamber was used in conjunction with a Cary 401 Vibrating Reed Electrometer for quantitation of radioactivity in gasses. The Rate-of-Charge method was used to determine the current through the ionization chamber with an applied voltage of 90 V. A standard curve was prepared using various amounts of $Ba^{14}CO_3$ with a specific activity of 1.16×10^3 dpm/mg (New England Nuclear, Boston). The $Ba^{14}CO_3$ was converted to gas using a carbon dioxide generator (Applied Physics Corporation, No. 3120000).

Quantitation of Gasses

Gasses were quantitated using a Packard 800 Series Gas Chromatograph. A 6 foot by 2 mm glass column was packed with Carboseive B (Supelco, Bellefonte, Pa.). Temperatures were inlet 100°C, column 70°C, detector 82°C, and outlet 96°C. Carrier gas flow rate was 30 ml of nitrogen per min. Detection was by thermal conductivity with a bridge current of 200 mA. The signal from the detector was connected to an Autolab Minigrator which was used to quantitate by comparison with standards.

RESULTS

Determination of Inhibitory Concentrations of Volatile Acids

Warburg vessels were gassed out with a 70% nitrogen: 30% carbon dioxide mixture. Fifty ml of enrichment culture (acetate, propionate, or butyrate) were added to each vessel with varying amounts of oxygen free volatile organic acid solution (the sodium salt of the acid corresponding to the enrichment). Manometric readings were taken hourly, and at the end of each experiment liquid samples were taken for volatile organic acid analysis. Figure 1 illustrates that for these enrichment cultures, volatile organic acid concentrations up to 50 mM are not inhibitory. All subsequent experiments were performed with volatile organic acid concentrations between 5 and 50 mM.

Effect of Molecular Hydrogen on Volatile Organic Acid Dissimilation

Carboxy-labeled ^{14}C volatile organic acids were used to measure the effect of molecular hydrogen on dissimilation rates of volatile organic acids. Labeled substrates were added to enrichments in Warburg vessels under an atmosphere of 70% nitrogen: 30% carbon dioxide. Gassing was continued and liquid samples were taken hourly. After 2 hr the atmosphere was replaced with a 70% hydrogen: 30% carbon dioxide mixture, and gassing continued for 2 additional hours, with liquid samples taken hourly. Figures 2 through 4 show that acetate dissimilation is slightly inhibited by molecular hydrogen, while propionate and butyrate dissimilation is completely shut off.

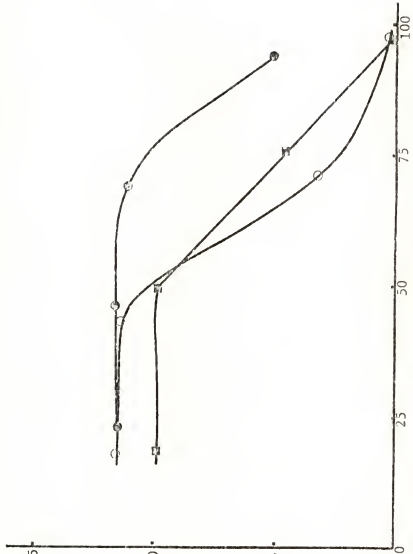

Fig. 1. Inhibition by volatile organic acids of gas production by methanogenic enrichments. With pH maintained at 6.9 to 7.0, enrichments were incubated at 30°C in the presence of varying concentrations of volatile organic acids. (● acetate enrichment with different concentrations of acetate, ○ propionate enrichment with different concentrations of propionate, ■ butyrate enrichment with different concentrations of butyrate)

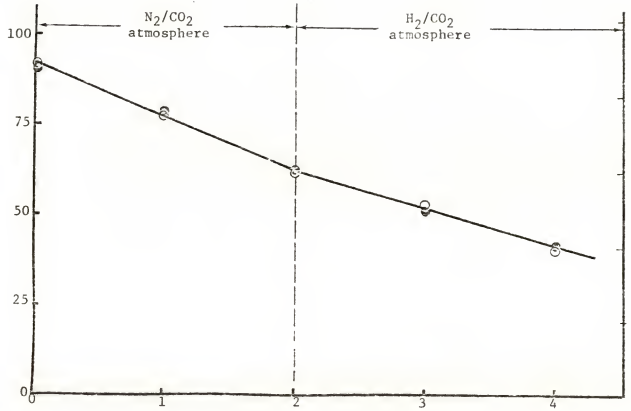

Fig. 2. Acetate utilization in the absence and presence of added molecular hydrogen. An acetate enrichment was incubated at 30°C with ^{14}C-labeled acetate in a 70% nitrogen: 30% carbon dioxide atmosphere. After 2 hr the atmosphere was changed to 70% hydrogen: 30% carbon dioxide while maintaining anaerobic conditions. (○ acetate concentration, ● radioactive label in the acetate fraction)

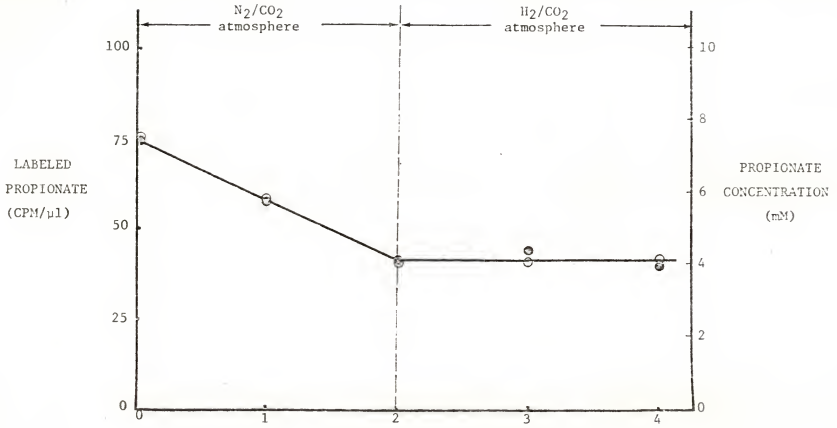

Fig. 3. Propionate utilization in the absence and presence of added molecular hydrogen. A propionate enrichment was incubated at 30°C with ^{14}C-labeled propionate in a 70% nitrogen: 30% carbon dioxide atmosphere. After 2 hr the atmosphere was changed to 70% hydrogen: 30% carbon dioxide while maintaining anaerobic conditions. (O propionate concentration, ● radioactive label in the propionate fraction)

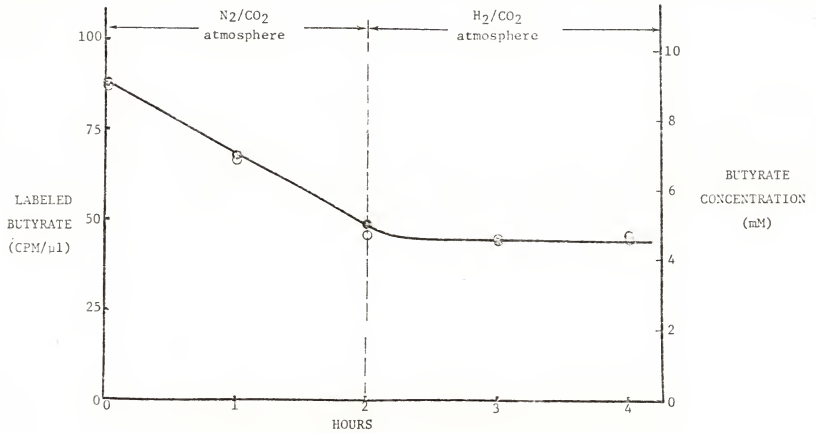

Fig. 4. Butyrate utilization in the absence and presence of added molecular hydrogen. A butyrate enrichment was incubated at 30°C with ^{14}C-labeled butyrate in a 70% nitrogen: 30% carbon dioxide atmosphere. After 2 hr the atmosphere was changed to 70% hydrogen: 30% carbon dioxide while maintaining anaerobic conditions. (○ butyrate concentration, ● radioactive label in the butyrate fraction)

Rates of Methanogenesis in the Absence and Presence of Added Molecular Hydrogen

Fifty ml samples of enrichment cultures were added anaerobically to Warburg vessels with 70% nitrogen: 30% carbon dioxide atmospheres, and gas production was measured hourly. After 6 hr the atmosphere was replaced with a 70% hydrogen: 30% carbon dioxide atmosphere, and gas uptake was measured every 10 min for 1 hr (see table 1). Gas samples taken at the end of this hour always contained within 8% of the methane expected from hydrogen uptake by the stoichiometry of the reduction of carbon dioxide with hydrogen (see equation 1). To determine that this methane was produced by carbon dioxide reduction, 50 ml aliquots of acetate enrichment were incubated in two Warburg vessels with atmospheres of 70% hydrogen: 30% carbon dioxide. Labeled sodium bicarbonate (^{14}C) was added, and flasks were incubated for 1 hr at 30°C while monitoring gas uptake manometrically. At the end of the incubation period, 10 ml of 1 M NaOH solution were added with a syringe. The NaOH solution caused the carbon dioxide to be absorbed by the liquid in the flasks, and the 10 ml volume approximately replaced the volume of gas absorbed, so the total volume of liquid plus gas remained about the same. Ten ml of gas was then removed for quantitation of radioactivity. Based on the specific activity of the bicarbonate and the amount of hydrogen utilized, the amount of labeled methane was calculated (assuming that all hydrogen utilized resulted in carbon dioxide reduction). In the duplicate flasks, 93% and 96% of the expected radioactivity was found

Table 1. Comparison of the ability of enrichments to produce gas from volatile organic acids (VOA) in a nitrogen-carbon dioxide atmosphere with their ability to utilize hydrogen in a hydrogen-carbon dioxide atmosphere.

Enrichment	Gas production rate from VOA (mm/hr)	H_2 utilization rate (mm/hr)	H_2 utilization / production from VOA
Acetate	17.7	36.7	2.1
Acetate	16.7	41.7	2.5
Acetate	15.4	39.2	2.5
Propionate	18.8	101.3	5.4
Propionate	17.0	76.7	4.5
Propionate	20.9	88.3	4.2
Butyrate	15.2	86.4	5.7
Butyrate	16.3	84.6	5.2
Butyrate	14.3	77.3	5.4

in the gas samples. This demonstrates that the vast majority of hydrogen absorbed in these experiments was used for carbon dioxide reduction.

Carbon Dioxide Sparging Experiments

A 500 ml gas scrubber was gassed out with 100% carbon dioxide (oxygen free), and 500 ml of enrichment culture or sludge were added. To prevent decreased pH when equilibrated with 100% CO_2, the carbon dioxide concentration in the enrichments were increased by adding 6 g $NaHCO_3$ while maintaining anaerobic conditions. The pH after equilibrium with 100% carbon dioxide was within the range of 6.8 to 6.9. Enrichments were maintained at room temperature; carbon dioxide was bubbled through the enrichments during the incubation. Effluent gas was collected and the carbon dioxide absorbed out using the alkaline gas collector. Collections were made at various sparge rates for each enrichment and the alkaline insoluble gasses analyzed for methane and hydrogen. In the case of the propionate and butyrate enrichments, feed to the enrichments was stopped 24 hr prior to its use in a sparging experiment. This allowed all substrate to be washed out or dissimilated, so that no detectable volatile organic acids were present. At the beginning of an experiment the desired substrate was added, so there was a minimum of dissimilation of other substrates during the experiments. The results with acetate, propionate, and butyrate as substrates are shown in figures 5 through 7.

To determine the quantity of methane and hydrogen remaining in the alkaline solution of the collector, 100% nitrogen was added to the collector through the septum after collected gas had been removed at the end of a sparging experiment. The nitrogen gas was shaken in the collector

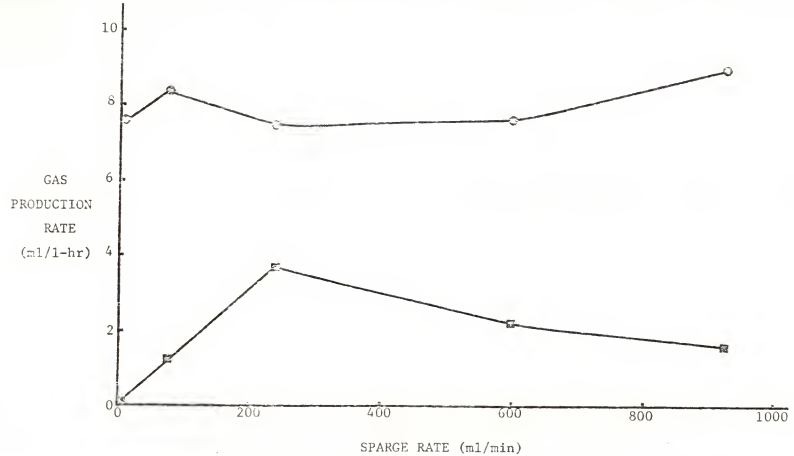

Fig. 5. Gas obtained by flushing an acetate utilizing propionate enrichment with 100% CO_2. Enrichments were incubated at room temperature with the pH within the range 6.8 to 6.9 and flushed with gas. The gas removed from solution in the enrichments was quantitatively analyzed to determine the rates of methane production and hydrogen production at various sparge rates. (● methane production rate, ◘ hydrogen production rate)

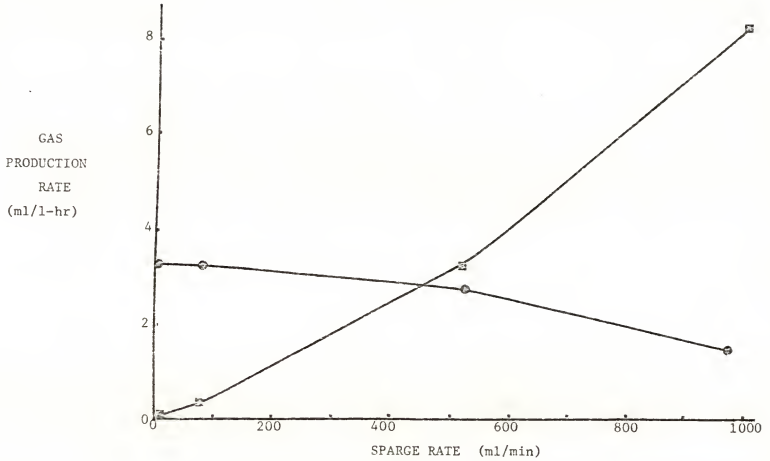

Fig. 6. Gas obtained by flushing a propionate utilizing propionate enrichment with 100% CO_2. Enrichments were incubated at room temperature with the pH within the range of 6.8 to 6.9 and flushed with gas. The gas removed from solution in the enrichments was quantitatively analyzed to determine the rates of methane production and hydrogen production at various sparge rates. (● methane production rate, ■ hydrogen production rate)

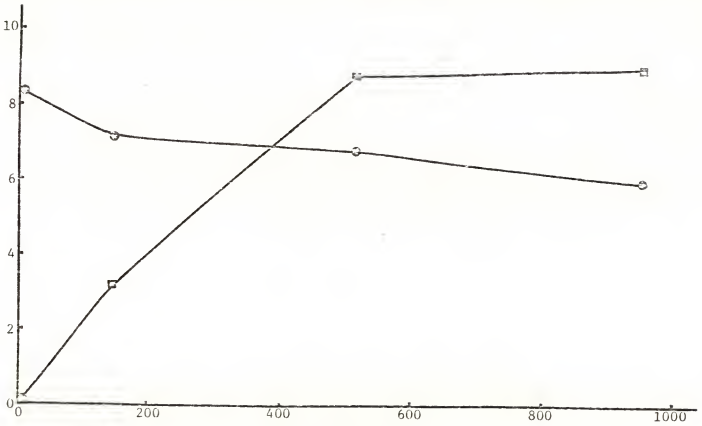

Fig. 7. Gas obtained by flushing a butyrate utilizing butyrate enrichment with 100% CO_2. Enrichments were incubated at room temperature with the pH within the range of 6.8 to 6.9 and flushed with gas. The gas removed from solution in the enrichments was quantitatively analyzed to determine the rates of methane production and hydrogen production at various sparge rates. (● methane production rate, ■ hydrogen production rate)

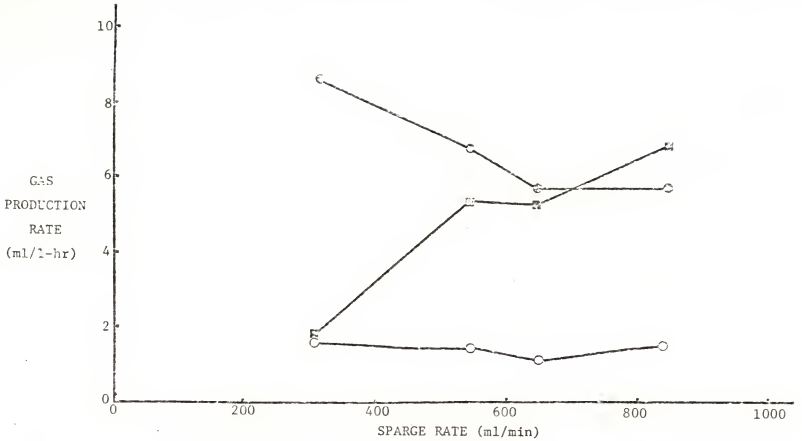

Fig. 8. Gas obtained by flushing a propionate utilizing propionate enrichment with 100% CO_2. Enrichments were incubated at room temperature with the pH within the range of 6.8 to 6.9, and were flushed with gas. The gas removed from solution in the enrichments was quantitatively analyzed to determine the rates of methane production and hydrogen production at various sparge rates. Methyl-^{14}C labeled sodium acetate was added for quantitation of methane derived from acetate. (● methane production rate, ■ hydrogen production rate, ○ methane production from acetate)

with the alkaline solution for 1 hr and removed for analysis. The gas that had remained in solution was calculated according to Henry's law and was found to represent less than 0.5% of the methane and less than 0.5% of the hydrogen produced during the sparging experiment. This was deemed insignificant, and routine analysis included only undissolved gasses.

Thirty liters from each tank of 100% carbon dioxide used for sparging experiments were absorbed using NaOH solution in the gas collector. No hydrogen and no methane was detected in the alkaline insoluble gas.

Sludge from the laboratory scale anaerobic digestor was also sparged, as well as sludge diluted 1:1 with sludge supernate. The supernate was prepared by adding sludge to a gassed out flask and cooling in an ice-water bath to halt gas production which might stir up sediment. Sludge was allowed to settle for 50 min and the supernate was siphoned off into the gas scrubber. It was then warmed to 35°C, and an equal volume of sludge was added from the digestor. As with the enrichment sparging experiments 6 g of $NaHCO_3$ was added to maintain pH at 6.8 to 6.9. Sludge sparging experiments were performed at 35°C and always began exactly 1 hr after the daily feeding of the digestors.

Pure Culture Isolations

Serial ten-fold dilutions were made from each enrichment in liquid media with a nitrogen and carbon dioxide gas phase. Solid media with hydrogen and carbon dioxide gas phase and solid media with nitrogen and carbon dioxide gas phase with 25 mM VOA (corresponding to the enrichment)

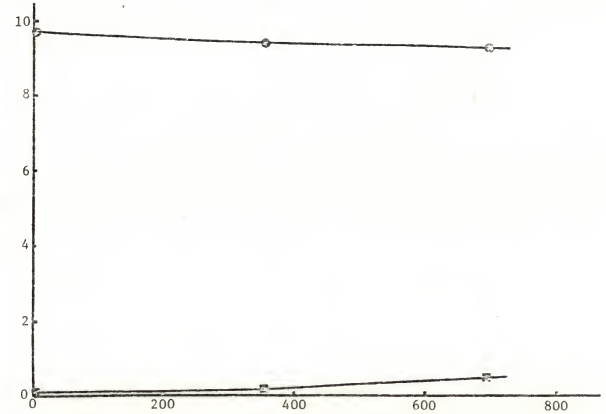

Fig. 9. Gas obtained by flushing anaerobically digesting sewage sludge with 100% CO_2. Sludge was incubated at 35°C within the pH range of 6.8 to 6.9. The dissolved gasses removed by flushing were quantitatively analyzed to determine the rates of methane production and hydrogen production. (● methane production rate, ■ hydrogen production rate)

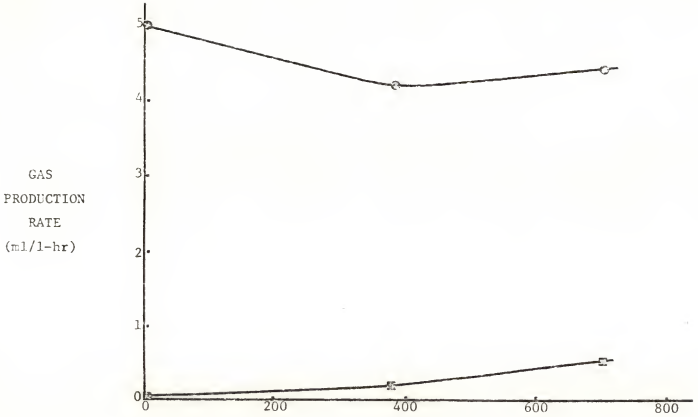

Fig. 10. Gas obtained by flushing dilute (1:1) anaerobically digesting sewage sludge with 100% CO_2. Sludge was mixed 1:1 with sludge supernate and incubated at 35°C within the pH range of 6.8 to 6.9. The dissolved gasses removed by flushing were quantitatively analyzed to determine the rates of methane production and hydrogen production. (● methane production rate, ■ hydrogen production rate)

added were inoculated from these dilutions. Also from each dilution of each enrichment three tubes of liquid media with hydrogen and carbon dioxide gas phase were inoculated. All tubes were incubated at 30°C for 4 weeks, with solid media incubated vertically and liquid media incubated horizontally.

The three tubes inoculated from each dilution of each enrichment were used in the enumeration of methanogenic bacteria in the enrichments. After incubation each tube was checked for methane production (greater than 10% methane was recorded as positive for methane production). This data was used to calculate numbers of methanogenic bacteria using the Most Probable Number (MPN) technique. This indicated methanogens populations of 1.1×10^7 per ml in the acetate enrichment, 4.4×10^8 per ml in the propionate enrichment, and 4.2×10^8 in the butyrate enrichment. It should be noted that the methanogens enumerated by this technique are only those which can utilize molecular hydrogen and carbon dioxide as methanogenic substrates.

Colonies picked from solid media with nitrogen and carbon dioxide gave no colonies with attempted subculturing in identical media. Colonies picked from solid media with hydrogen and carbon dioxide gas yielded 16 isolates after subculturing. These were isolated from original dilutions of 10^{-7} or greater. All isolates were rods of varying size, none of which were able to utilize propionate or butyrate under nitrogen and carbon dioxide or hydrogen and carbon dioxide gas phase.

DISCUSSION

Dissolved hydrogen gas in methane fermentations occurs at a concentration too low to be measured directly. The hydrogen uptake experiments demonstrate that hydrogen can be rapidly oxidized by propionate and butyrate enrichments. The sperging experiments demonstrate an ability of these organisms to produce hydrogen. It is possible that large amounts of hydrogen are produced and immediately utilized so that the pool size of molecular hydrogen remains small. Thus hydrogen concentrations too low to detect in anaerobic digestors do not disprove its role as an important methanogenic intermediate.

The dissimilation of propionate and butyrate is inhibited by molecular hydrogen, so one would expect the level of hydrogen to remain very low, assuming these volatile acids to be the major source of hydrogen. This inhibition can be predicted by the thermodynamics of the production of hydrogen from propionate and butyrate.

It has been shown that acetate can be converted directly to methane and carbon dioxide. Therefore, the behavior of these enrichments dissimilating acetate serves as a control to which propionate and butyrate dissimilation are compared. But because acetate is an intermediate in the breakdown of propionate and butyrate, the conversion of acetate to methane and carbon dioxide occurs during all the sparging experiments.

In the sparging experiments dissolved hydrogen was stripped from solution by allowing it to diffuse into bubbles of carbon dioxide. To remove significant amounts of hydrogen present at such low concentrations, large volumes of carbon dioxide were moved through the

liquid. The sparged gas was then greatly reduced in volume by absorbing out acidic gasses (predominantly carbon dioxide), thereby increasing the concentrations of gasses removed from solution to levels that are easily quantitated. More hydrogen is removed at faster sparge rates because there are more bubbles in the liquid at any given time (hence a larger surface area for diffusion), and because the concentration of hydrogen in the gas bubbles remains lower when larger amounts of carbon dioxide are moving through the liquid, giving a greater concentration differential.

Hydrogen production from enrichments fermenting propionate and butyrate is readily demonstrated. A propionate enrichment was shown to produce hydrogen when propionate and acetate were present as substrates, but not when acetate alone was present. The smaller amounts of hydrogen in sparged gas from sludge may be due to the high levels of solids present. Bacteria adhering to solids are in much closer proximity to other bacteria than they would be if they were dispersed in the liquid, and hydrogen may be utilized by adjacent bacteria before it can diffuse out of the intercellular spaces at the surface of the solids.

The hydrogen uptake experiments showed that methanogenic fermentations enriched with propionate or butyrate retain the ability to utilize hydrogen. When a substrate which can be converted to methane and carbon dioxide directly (acetate) is used, the ability of the enrichment to utilize hydrogen diminishes because hydrogen oxidizers are washed out. These data do not rule out a role for molecular hydrogen in the conversion fo acetate to methane and carbon dioxide, but in these enrichments the predominant pathway for acetate

dissimilation does not include molecular hydrogen as an intermediate.

In summary, the evidence that molecular hydrogen is an intermediate in propionate and butyrate dissimilation is: Enrichments are able to produce large amounts of hydrogen from these substrates, they are able to convert large amounts of hydrogen to methane in the presence of carbon dioxide, and in enriching cultures with propionate or butyrate one also enriches for hydrogen oxidizing methane producing bacteria which cannot themselves utilize these substrates.

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BIOGRAPHICAL SKETCH

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He is married to the former Jane Ann Emslie. He is a member of American Association for the Advancement of Science, American Society for Microbiology, Society for Industrial Microbiology, Water Pollution Control Federation, and the International Association for Water Pollution Research.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Paul H. Smith, Chairman
Professor of Microbiology and Cell Science

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James F. Preston
Associate Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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This thesis was submitted to the Graduate Faculty of the Department of Microbiology and Cell Science in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1977

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